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A Genome-Wide Analysis of DNA Methylation and Fine Particulate Matter Air Pollution in Three Study Populations: KORA F3, KORA F4, and the Normative Aging Study

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ABSTRACT

Background: Epidemiological studies have reported associations between particulate matter (PM) concentrations and cancer, respiratory, and cardiovascular diseases. DNA methylation has been identified as a possible link but so far it has only been analyzed in candidate sites.

Objectives: To study the association between DNA methylation and short- and mid-term air pollution exposure using genome-wide data, and identify potential biological pathways for additional investigation.

Methods: We collected whole blood samples from three independent studies, KORA F3 (2004-05) and F4 (2006-08) from Germany and Normative Aging Study (1999-2007) from the US, and measured genome-wide DNA methylation proportions with the Illumina 450k BeadChip. PM concentration was measured daily at fixed monitoring stations and three different trailing averages were considered and regressed against DNA methylation: 2-day, 7-day and 28-day. Meta-analysis was performed to pool the study-specific results.

Results: Random-effect meta-analysis revealed 12 CpG (cytosine-guanine dinucleotide) sites as associated with PM concentration (one for 2-day average, one for 7-day and ten for 28-day) at a genome-wide Bonferroni significance level ($p<=7.5E-8$); 9 out of these 12 sites expressed increased methylation. Through estimation of I-squared statistics for homogeneity assessment across the studies, four of these sites (annotated in *NSMAF*, *C1orf212*, *MSGN1*, *NXN*) showed $p>0.05$ and $I^2<0.5$: the site from the 7-day average results and 3 for the 28-day average. Applying False Discovery Rate, p-value<0.05 was observed in 8 and 1819 additional CpGs at 7- and 28-day average $PM_{2.5}$ exposure respectively.

Conclusion: The PM-related CpG sites found in our study suggest novel plausible systemic pathways linking ambient particulate matter exposure to adverse health effect through variations in DNA methylation.

INTRODUCTION

Ambient air pollution has been associated with total mortality, as well as cardiorespiratory disease morbidity and mortality (Brook et al. 2010; Hoek et al. 2013). Recently, association between long-term exposure to ambient air pollution, benzene and nitrogen dioxide, and lung cancer has been reported in North America and Europe (Puett et al. 2014; Raaschou-Nielsen et al. 2013; Villeneuve et al. 2014). Especially fine particulate matter ($PM_{2.5}$: particulate matter smaller than 2.5 μm) is believed to be responsible for the associations. The WHO estimates 3.7 million premature deaths worldwide in 2012 due to ambient air pollution (WHO 2014).

Findings based on animal models suggest that oxidative stress and inflammatory responses initiated upon deposition of fine particulate matter in the alveoli may be key pathophysiologic mechanisms linking exposure to ambient fine particles to both respiratory and cardiovascular diseases in humans (Cassee et al. 2013). Oxidative stress and inflammation have also been proposed as underlying mechanisms linking PM and cancer, including lung cancer (Soberanes et al. 2012; Zhao et al. 2013). Despite these evidences, the extent to which systematic effects are elicited by ambient particles, and the detailed pathways activated are still under debate (Peters 2012). Novel molecular approaches such as genome-wide methylation assays allow a hypothesis-free assessment of changes in the regulation of blood leukocytes, involved in CVD development (Baccarelli and Bollati 2009).

Changes in global methylation as well as in candidate genes (Bind et al. 2014) were observed in individuals with high occupational exposure such as foundry workers in a small study (Tarantini et al. 2009) or in response to ambient PM concentrations few hours before the study visit

(Baccarelli et al. 2009). However it is difficult to determine the exact time window associated with methylation.

Genome-wide methylation assays allow taking advantage of advances in biological technologies in epidemiological studies (Christensen and Marsit 2012) and studying in particular the role of ambient fine particle concentrations in the days and weeks before biosample collection.

The objective of the analyses presented here is to identify and investigate DNA methylation at CpG sites in association with short- and mid-term $PM_{2.5}$ ambient exposure. In addition, we consider biological pathways that might mediate associations between $PM_{2.5}$ and health outcomes, based on the specific CpG sites identified.

METHODS

Three independent cohort studies formed the basis for the analyses presented here. Uniform methods were applied for fine particle measurements and methylation methods.

Study populations

KORA F3 and F4 cohorts are follow-up studies from the previous KORA S3 and S4, two surveys enrolled in the region of Augsburg (South Germany) by sampling all inhabitants with German nationality aged 25-74 in accordance with principles of the Declaration of Helsinki. Respectively, they included 3,988 and 4,227 participants and data were collected between 2004/05 (F3) and 2006/08 (F4) according to standardized operating procedures. Exhaustive information about these two studies has been described previously (Holle et al. 2005; Wichmann et al. 2005). Methylation profiles were evaluated for a total of 500 KORA F3 participants and 1,799 F4 participants. No sample overlap appears between F3 and F4 and all participants

supplied written informed consent and they were approved by the Ethics Committee of the Bavarian Medical Association.

The Veteran Affairs (VA) Normative Aging Study (NAS) is an ongoing longitudinal study of aging established in 1963, details of which have been published previously (Bell et al. 1972). Briefly, the NAS is a closed cohort of 2,280 male volunteers from the Greater Boston area aged 21–80 years at entry, who enrolled after an initial health screening determined that they were free of known chronic medical conditions. The present study was approved by the Department of Veterans Affairs Boston Healthcare System, and written informed consent was obtained from subjects prior to participation. They have been reevaluated every 3–5 years by using detailed on-site physical examinations and questionnaires. Blood samples were provided from 657 participants and for most of them a second sample was drawn (1,119 samples in total) between 1999 and 2007.

We restricted the current analysis to white participants (n= 657) in order to increase comparability across the studies.

Profiling of DNA Methylation

We used the Illumina 450k Beadchip (following the Illumina Infinium HD Methylation Protocol) to assess DNA methylation in more than 480,000 CG dinucleotide (CpG) methylation sites throughout the entire genome (Zeilinger et al. 2013). Detailed validation and evaluation of this technology are provided by Sandoval et al. (Sandoval et al. 2011) and Dedeurwaerder et al. (Dedeurwaerder et al. 2011). Outputs of the chip are ß values that represent the percentage of methylation for every CpG target. Since the microarray measures each CpG site with either of

two technically distinct types of probes, the distribution of resulting methylation values differs. Here the approach used to preprocess the data: 1- data quality: removal of records according to functional beads, detection p-value and SNP frequency; 2- data correction: background subtraction and dye bias adjustment; 3: probe type adjustment: Beta-mixture quantile normalization (BMIQ, (Teschendorff et al. 2013)). Normalization process was chosen based on review papers (Marabita et al. 2013; Wu et al. 2014).

Environmental measurement

Specifically, in KORA, $PM_{2.5}$ mass concentration in ambient air and temperature were measured hourly at one monitoring station approximately 1 km south-east of the city center of Augsburg for the length of the whole study period 2004-2008 (Birmili et al. 2010; Pitz et al. 2008) with the Tapered Element Oscillating Microbalance (TEOM model 1400A device Rupprecht and Patashnick). 44 days were missing in KORA in 2004-2008 and eventually excluded from calculation of trailing averages.

In NAS, ambient $PM_{2.5}$ concentration was monitored downtown Boston 1 km from the VA medical center. We measured hourly $PM_{2.5}$ concentrations with the same device as in Augsburg. Hourly temperature data were obtained from the Boston Logan airport weather station (12 km from the medical center). Sampling, processing of samples, analysis and reporting were conducted according to standard operating procedures (Dockery et al. 2005). Missing hourly concentration data for $PM_{2.5}$ were imputed using regression modeling, including a long term time trend, day of week, hour of day, temperature, relative humidity, barometric pressure and nitrogen dioxide concentrations (NO_2) as predictors.

Statistical Analysis

An Epigenome Wide Analysis Study (EWAS) was conducted in each of the three studies. Based on previous knowledge (Baccarelli et al. 2009; Bruske et al. 2010; Steenhof et al. 2014; Zeilinger et al. 2013) we defined a priori model with the following covariates: age, personal income (education years for NAS, in which information on income was not available), alcohol intake, BMI, temperature (trailing average always matching with the PM exposure window) and the proportion of five white blood cell types: monocytes, B Cells, CD8 T cells, CD4 T cells, NK (estimated with a method developed by Houseman et al, (Houseman et al. 2012)) as continuous and sex, smoking status (never, former, current and passive - only for KORA - smokers), day of the week and season (according to the astronomical definition) as categorical. Complete variable coverage is in Table 1. In order to investigate the association between short- and mid-term $PM_{2.5}$ and DNA methylation, we considered three different averaging periods (2-, 7- and 28-day) backwards starting from the day of the visit, decided a priori based on Bind et al. (Bind et al. 2014), Schwartz (Schwartz 2000) and Rückerl et al. (Rückerl et al. 2007). For KORA, multivariable linear regression models were used to investigate the association between $PM_{2.5}$ exposure and methylation values:

$$Y_i = \beta_0 + \beta_1\, PM_{2.5i} + \beta_2\, Temperature_i + \beta_3\, X_{3i} + \ldots + \beta_p X_{pi} + \varepsilon_i \qquad [1]$$

Where Y_i is the methylation measurement for subject *i*, β_0 is the intercept, β_1 and β_2 are the coefficients of the trailing average values for exposure and temperature during the specific time window, X_{3i} to X_{pi} are the *p–2* covariates and ε_i is the error. Effect estimates represent the difference in methylation associated with a 10 $\mu g/m^3$ increase in $PM_{2.5}$. For NAS data, we fitted generalized mixed-effect models in order to account for the repeated measurements; time-variant

covariates were assessed at both first and second visit and a random participant effect (u_i) was applied in order to take the data collection at two different time points into account:

$$Y_{it} = \beta_{0t} + \beta_1 PM_{2.5it} + \beta_2 Temperature_{it} + \beta_3 X_{3it} + \ldots + \beta_p X_{pit} + u_i + \varepsilon_{it} \quad [2]$$

Finally, we pooled cohort-specific estimates, when available for all three studies, for each exposure window by random-effect meta-analysis (428,415 CG targets). Bonferroni threshold (fixed at 7.5E-08) and False Discovery Rate (FDR, (Benjamini and Hochberg 1995)) with Benjamini-Hochberg criterion was used to adjust fixed-effect p-values for multiple comparisons. I-squared test on fixed-effect estimates have been used to assess heterogeneity and CpGs with p-values > 0.05 and $I^2 < 0.5$ were labeled as homogenous. Finally, a number of sensitivity analyses were performed. We repeated the a priori models with additional adjustment for average annual exposure during the year before the visit to assess potential confounding by long-term exposure. In addition, we ran models adjusted only for age and sex, and models adjusted only for age, sex, and white blood cell proportions. All analyses were performed using statistical software R, Version 2.14. Residual plots of significant CpGs were used to check whether the identified CpGs where driven by outliers. To discard these values we used a rule of thumb based on biological knowledge. DNA methylation in the 0-1 range can be divided in hypo-, hemi- and hyper-methylation with ranges [0 – 0.35], [0.35 – 0.65] and [0.65 – 1] respectively. Once selected the CpGs with very high residuals (absolute value above 0.25), identified the methylation segment where the mean was located and discarded all the values out of it, the analysis was repeated.

Functional analysis of the identified genes has been performed via a web interface (Warde-Farley et al. 2010).

RESULTS

Data from three independent cohort studies were available (Table 1). Specifically, cross-sectional data from two independent sub-samples of the KORA study (KORA F3, n=500 participants and F4, n=1,799) and cohort data collected as part of the Normative Aging Study (NAS, n=657) formed the basis of the analyses presented here. The NAS included only men with an average age of 72 years while KORA F3 and F4 participants (52 and 49% of males) were on average 53 and 61 years old. While body mass index was rather similar, substantial differences were observed for years of education (mean of 15.1 in NAS vs 11.7 and 11.5 in KORA F3 and F4) and alcohol consumption (19.7% of drinkers for NAS vs 59.2 and 57.7% for KORA F3 and F4). Regarding smoking, KORA F3 consisted mostly of never and current smokers, KORA F4 of former and current while around two thirds of NAS participants were former smokers. Whereas NAS has on average lower particle concentration the day before the visit, it showed higher average temperature than the KORA studies. During the study period, $PM_{2.5}$ exceeded the daily US EPA standard of 35 $\mu g/m^3$ 7.5% of the days in F3 (2004-05), 5.9% in F4 (2006-08) and 2.9% in NAS (1999-2007). Consistent methylation averages were observed between the three studies with relatively small standard deviations (Table 2-3).

The meta-analyses identified genome-wide significant ($p < 7.5E-08$) associations between $PM_{2.5}$ exposure averaged over 2 days up to 4 weeks and single CpG-sites (Figure 1). DNA methylation at one CpG site (cg25575464 within *NEURL4*, chromosome 17) reached genome-wide significance ($p < 7.5E-08$) in association with 2-day trailing average $PM_{2.5}$, with a positive association indicating higher methylation at 10 $\mu g/m^3$ increase in exposure (Table 2, Supplemental Material Figure S1). Although study-specific associations were all positive, there

was significant heterogeneity among the studies. For 7-day average $PM_{2.5}$ concentration, the association with one CpG site, cg19963313 (*NSMAF*, chr 8) reached genome-wide significance (Table 2) and study-specific estimates were positive and homogenous (I^2=0.0, p-value 0.59) (Figure 2). Associations between 7-day $PM_{2.5}$ and cg02608596 (*MPND*, chr 9) also were positive and homogeneous among the three studies, though the p-value was slightly above the alpha level for genome-wide significance (p = 7.69E–08). Cg02608596 and 7 additional CpGs had FDR p-values < 0.05 for 7-day $PM_{2.5}$, including cg25575464, which also was associated with 2-day $PM_{2.5}$ (Table 2). Associations between 7-day $PM_{2.5}$ and the additional CpGs were heterogeneous among the study sites in three cases and homogeneous in four cases. No additional CpG sites were identified as associated with 2-day $PM_{2.5}$ based on FDR < 0.05. Associations between ten CpGs and 28-day average exposure to $PM_{2.5}$ reached genome-wide significance, including 3 with lower methylation [cg16308101 (*SERBP1*, chr 1), cg13169286 (no annotated gene, chr 10), and cg20680669 (*MN1*, chr 22)] and 7 with higher methylation [cg23276912 (*C1orf212*, chr 1), cg03455255 (*TSPYL6*, *ACYP2*, chr 2), cg11046593 (*MSGN1*, chr 2), cg04423572 (*ACVR2B-AS1*, chr 3), cg19215199 (*ZMIZ1*, chr 10), cg13527922 (*F2*, chr 11), cg26003785 (*NXN*, chr 17)] (Table 3). Study-specific associations were homogenous for cg23276912, cg11046593, and cg26003785 (Figure 3), but heterogeneous for the other CpGs (Supplemental Material, Figure S1). When we considered all associations with FDR p < 0.05, a total of 1,829 CpG sites were associated with 28-day average $PM_{2.5}$ (Supplemental Material, Excel File S1), including five in genes with at least one Bonferroni significant CpG (also shown in Table 3): cg16856342 (*SERBP1*, chr 1), cg02795981 (*ZMIZ1*, chr 10), cg24101979 and cg26283240 (*NXN*, chr 17) and

cg06004017 (*MN1*, chr 22). One CpG with a significant FDR for 28-day $PM_{2.5}$ reached genome-wide significance for 7-day $PM_{2.5}$ (cg19963313, *NSMAF*, chr 8).

Sensitivity Analysis

Genome-wide significant CpGs at 28-day were also adjusted for long-term exposure and resulted in consistent estimates and p-values, except for cg20680669 which estimate moved from a ß = -0.0049 with p = 2.09E-08 (without long-term) to ß = -0.0020 with p = 2.36E-03 and cg26003785 which moved from ß = 0.0038 with p = 9.53E-09 to ß = 0.0033 with p = 1.10E-06 (Supplemental Material, Table S2). Furthermore, we checked for potential influences of outliers (Supplemental Material, Figures S2-S4). Cg11046593 was of concern in these plots and 22 values were excluded for F4, 1 for F3 and 12 for NAS. However, the association remained significant: the estimate moved from 0.016 to 0.012 and the p-value from 1.12E-08 to 5.48E-08.

DISCUSSION

This meta-analysis of three cohort studies identified twelve CpGs genome-wide significantly associated with ambient fine particulate matter concentrations at different exposure times based on Bonferroni corrections. Based on previous knowledge (Bind et al. 2014; Rückerl et al. 2007; Schwartz 2000), we considered three different cumulative exposure windows: 2, 7 and 28 days and we observed that the number of associations was larger for the longest exposure window. Nine CpG sites displayed increased methylation and three decreased methylation after exposure to fine ambient particle concentrations. All identified methylation sites displayed little overall variation (average coefficient of variation: 15%) within the study populations. Four of them manifested homogeneous changes across the three different studies. Applying FDR, 7 and 1819 additional CpGs were found significant at 7- and 28-day average $PM_{2.5}$ exposure respectively.

The CpG site (cg19963313) identified with the 7-day trailing average showed homogeneity among the studies. Cg19963313 is positioned in the gene *NSMAF* that is linked with the 55kD tumor necrosis factor receptor since it encodes a WD-repeat protein which binds its cytoplasmic sphingomyelinase activation domain (Montfort et al. 2010). Moreover, it participates in the same reaction within a pathway as *SMPD2* (Wu et al. 2010), which has been demonstrated in primary cells to be linked to oxidative stress (Byon et al. 2008; Jana and Pahan 2007). Furthermore, it has been identified in cellular response to hyperosmolar stress (Robciuc et al. 2012). Hyperosmolarity is well known to impose remarkable stress on membranes, especially the ones that are in direct contact with the environment (Hallows et al. 1996), but it has never been associated with air pollution.

Furthermore we identified three CpG sites significantly and homogeneously associated with the 28-day average exposure to fine particle: cg26003785, cg11046593 and cg23276912 annotated to *NXN*, *MSGN1* and *C1orf212* respectively, which are protein-coding genes.

Specifically, *NXN* has been observed as partner of phosphofructokinase (PFK) 1, a glycolytic enzyme, reported as contributor for systemic metabolic conditions and also cancerous processes (Mor et al. 2011; Yi et al. 2012).

Increased methylation was detected at cg11046593, located in the promoter of *MSGN1*, that - when methylated - has been shown to lead to transcriptional repression (Jones and Takai 2001). Domain databases also determined shared protein domain with *AHR* (Aryl Hydrocarbon Receptor) and *ARNT* (Aryl Hydrocarbon Receptor Nuclear Translocator), involved in regulation of inflammatory processes implicated in multi-factorial diseases like pulmonary disorders

(Scrivo et al. 2011; Ukena et al. 2010). It was found that these two genes regulate chemokine-responses mostly relating *AHR* and *ARNT* to the nuclear factor-kB family (NF-kB) where the p65/p50 dimer is pivotal in the regulation of the inflammatory responses (Ovrevik et al. 2014; Vogel and Matsumura 2009). *AHR* and particulate matter exposure have already been associated through nongenotoxic events and Th17 polarization (Andrysik et al. 2011; van Voorhis et al. 2013), but here we observed a epigenetic factor as possible mediator. Even without a direct association, the discovery of *MSGN1* provides a novel hypothesis in the path between exposure to endogenous factors and immunological system responses and future studies are needed to verify and eventually clarify the possible role of *ARNT*.

Temporal Variation within Short- and Mid- Term Range

For cumulative exposures over 28 days, ten CpG sites were genome-wide significant. Larger datasets are needed to better understand the optimal exposure time window and to confirm a hypothesis, that it may be CpG site-specific. The cases of cg25575464 (Bonferroni significant at 2-day, FDR significant at 7-day and non-significant at 28-day average) and cg19963313 (non-significant at 2-day, Bonferroni significant at 7-day and FDR significant at 28-day average), might be consistent with the hypothesis regarding CpGs associated at shorter time periods but not over longer time.

One of the genes we highlighted, *ZIMZ1*, has already been connected to skin tumors in mouse models (Rogers et al. 2013) and our results, independently, link it to PM exposure via DNA methylation, reinforcing the hypothesized role of epigenetics in the pathways to tumor development (Laird 2005).

We observed mostly positive effect estimates, in this genome-wide methylation study, in contrasts with previous results (Guo et al. 2014) that observed a negative association between short-term PM exposure and DNA methylation in tandem repeats. Zeilinger et al. (Zeilinger et al. 2013) observed decreased methylation as consequence of active smoking in a cross-sectional study. Their most striking and significant CpG belongs to *AHRR* that repress *AHR* and we observed increased methylation in a gene that shares protein domain with *AHR*. Possible relations and implications need to be verified in the future.

Strengths and Limitations

The data presented here combines evidence from three independent studies, each at least considering data of 500 participants, a paramount element to identify differentially-methylated CpG sites that have a very little variability. We also adjusted our models for important variables that may otherwise confound the effect of associations with ubiquitous exposures such as ambient air pollution. Finally, we used daily averages of temperature to calculate the same trailing averages and apply appropriate adjustment for weather conditions. We performed a number of sensitivity analysis. Overall, the results of a priori chosen model were considered a conservative estimate. The observed hits between $PM_{2.5}$ and CpG sites were independent of long-term exposure at the residence and were not influenced by potential outliers. This study has also limitations. There is a consensus in the scientific community that a background station measuring particulate matter with aerodynamic diameter ≤ 2.5 μm ($PM_{2.5}$) mass concentrations could be regarded as representative for larger urban areas (Monn 2001). Considering that no coal power plant is in operation in proximity of the participants and only a small percentage of them live close to a major road we had to rely on ambient air pollution measurements since personal

exposures were not available. Measurement error from using a single site in this study is expected to result in primarily Berkson-type measurement error (Zeger et al. 2000), which would bias the standard errors, but not the estimated associations. We also acknowledge that the study included only whites, and generalizability to other populations is uncertain. While KORA was cross-sectional, the NAS study assessed the role of ambient particles longitudinally on time. Nevertheless, we had not comparable exposure estimates available to assess the long-term effect of ambient particles. Finally, the Illumina 450k does not completely cover the entire epigenome.

CONCLUSIONS

In conclusion, in this epigenome-wide investigation of CpG dinucleotide methylation, we highlighted several CpG sites associated with cumulative exposure to ambient particles up to a month. The trend of significance level of our results tends to increase with the length of the averaging period and the majority shows an increase in methylation. The identified genetic loci suggest novel biological pathways that may link ambient particulate matter to health outcomes such as tumor development and also gene regulation, inflammatory stimuli, pulmonary disorders and glucose metabolism. Future mechanistic studies are needed to establish whether these epigenetic changes could potentially explain the evidence found for ambient fine particles and lung cancer incidence.

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Table 1. Descriptive statistics of the study participants in the KORA F3, KORA F4 and US Veteran Affairs Normative Aging Study (VA)

Variables	Mean ± SD / N (%)		
	KORA F3 (n=500, 2004-05)	**KORA F4 (n=1,799, 2006-08)**	**NAS baseline[a] (n=657)**
Participants Characteristics			
Males	260 (52.0)	887 (49.3)	657 (100)
Age, years	53.12 ± 9.6	60.92 ± 8.9	72.44 ± 6.9
BMI[b], kg/cm^2	27.15 ± 4.5	28.15 ± 4.8	28.07 ± 4.1
Monthly Income, euro	1104.8 ± 583.9	1159.84 ± 556.6	*
Education, years	11.7 ± 2.8	11.5 ± 2.5	15.07 ± 2.9
Drinkers[c]	296 (59.2)	1038 (57.7)	130 (19.7)
Alcohol Consumption, g/day	16.11 ± 19.6	15.49 ± 20.4	*
Smoking			
Never Smokers	226 (45.2)	226 (12.6)	188 (28.6)
Former Smokers	11 (2.2)	782 (43.5)	446 (67.9)
Current Smokers	232 (46.0)	753 (41.9)	23 (3.5)
Passive Smokers (either Former or Never)	11 (2.2)	36 (2.0)	*
Missing	20 (4.4)	2 (0.0)	0 (0.0)
Environmental Exposure (mean of the daily average of the day before the visit)			
$PM_{2.5}$[d], µg/m^3	20.0 ± 11.6	14.2 ± 10.2	10.6 ± 7.1
Percentiles (25th, 50th, 75th)	14.0, 17.7, 25.9	6.7, 12.2, 18.8	6.3, 9.0, 13.2
Temperature, °C	7.1 ± 7.5	8.7 ± 6.6	12.5 ± 8.5
Percentiles (25th, 50th, 75th)	0.9, 7.9, 13.2	3.9, 7.5, 13.1	6.4, 12.7, 19.8

[a] First time blood sample was collected (time window: 1999-2007)

[b] Body Mass Index

[c] Participants with at least 2 drinks per week

[d] Particulate Matter smaller than 2.5 µm

* Data not available

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Table 2. Characteristics of the CpG sites from meta-analyses of 2- and 7-day trailing averages, significant with Bonferroni or FDR methods

Name	CHR[a]	Reference Gene Name	Methylation level Illumina Beta, Mean ± SD				Fixed-effect Regression Coefficient[b]	Sig.[c]	FDR[d]	I^2 (%)	Sig. I^2
			F3	F4	NAS	Mean					
Trailing 2-day average $PM_{2.5}$[e]											
cg25575464	17	*NEURL4*	.03 ± .01	.02 ± .01	.01 ± .01	.02 ± .01	0.00082	4.69E-08	0.005	91.0	<0.001
Trailing 7-day average $PM_{2.5}$[f]											
cg04078416	3	*CCDC12*	.05 ± .01	.05 ± .01	.02 ± .01	.04 ± .01	0.0001	4.19E-07	0.027	0.0	0.52
cg15996282	5	*LMBRD2*; *SKP2*	.04 ± .01	.04 ± .03	.02 ± .01	.04 ± .02	0.0020	7.25E-07	0.010	0.0	0.55
cg00402617	8	*YWHAZ*	.07 ± .01	.06 ± .02	.03 ± .01	.06 ± .02	0.0002	1.29E-07	0.018	62.3	0.07
cg19963313[g]	8	*NSMAF*	.04 ± .01	.03 ± .01	.02 ± .01	.03 ± .01	0.0018	2.49E-08	0.016	0.0	0.59
cg15883382	10	NA[h]	.04 ± .01	.05 ± .01	.02 ± .01	.04 ± .01	0.0001	8.43E-07	0.040	62.2	0.07
cg09225537	15	*MAG*	.03 ± .01	.02 ± .01	.01 ± .01	.02 ± .01	0.0001	4.44E-07	0.027	0.0	0.75
cg08757611	17	NA[h]	.03 ± .01	.03 ± .01	.02 ± .01	.03 ± .01	9.70E-05	2.15E-07	0.018	0.0	0.68
cg25575464	17	*NEURL4*	.03 ± .01	.02 ± .01	.01 ± .01	.02 ± .01	0.0001	1.76E-07	0.018	87.6	<0.001
cg02608596[g]	19	*MPND*	.04 ± .01	.03 ± .02	.02 ± .01	.03 ± .02	0.0017	7.69E-08	0.010	4.8	0.35

[a] CHR: chromosome

[b] Estimated difference in methylation for a 10 μg/m^3 increase in $PM_{2.5}$ adjusted for sex, age, income (education years for NAS, in which information on income was not available), smoking status, alcohol intake, BMI, temperature (moving average always matching

with the PM exposure window), day of the week, season and the proportion of five estimated white blood cell types: Monocytes, B Cells, CD8 T Cells, CD4 T Cells, NK

[c] P-values, Bonferroni significance level at 7.5E-08

[d] FDR: False Discovery Rate with Benjamini-Hochberg method, significance level at 0.05

[e] 2-day Trailing average starting from the day of the visit

[f] 7-day Trailing average starting from the day of the visit

[g] Shown in Figure 2

[h] NA: no annotated gene

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Table 3. Characteristics of the CpG sites from meta-analysis of 28-day trailing average, significant with Bonferroni method, or FDR significant and located in a gene with another CpG that meets genome-wide significance, or FDR significant and Bonferroni significant at shorter time-window

Name	CHR[a]	Reference Gene Name	Methylation level Illumina Beta, Mean ± SD				Fixed-effect Regression Coefficient[b]	Sig.[c]	FDR[d]	I^2 (%)	Sig. I^2
			F3	F4	NAS	Mean					
cg16308101	1	*SERBP1*	.45 ± .03	.46 ± .03	.44 ± .03	.45 ± .03	-0.0076	2.86E-08	0.002	91.3	<0.001
cg16856342[e]	1	*SERBP1*	.46 ± .02	46 ± .02	.38 ± .02	.44 ± .02	-0.0061	1.74E-07	0.003	1.4	0.36
cg23276912[f]	1	*C1orf212*	.87 ± .03	.89 ± .03	.86 ± .04	.90 ± .03	0.0073	4.56E-08	0.002	25.9	0.26
cg03455255	2	*TSPYL6*; *ACYP2*	.90 ± .02	.92 ± .01	.93 ± .02	.92 ± .02	0.0047	1.86E-08	0.001	61.8	0.073
cg11046593[f]	2	*MSGN1*	.80 ± .05	.83 ± .09	.86 ± .07	.83 ± .08	0.016	1.12E-08	0.001	46.1	0.16
cg04423572	3	*ACVR2B-AS1*	.70 ± .04	.74 ± .04	.74 ± .03	.73 ± .04	0.013	7.26E-09	0.001	97.3	<0.001
cg19963313[g]	8	*NSMAF*	.04 ± .01	.03 ± .01	.02 ± .01	.03 ± .01	0.0024	4.12E-07	0.005	0.0	0.90
cg13169286	10	NA[h]	.55 ± .03	.59 ± .07	.51 ± .06	.57 ± .06	-0.013	6.21E-08	0.003	85.4	<0.001
cg02795981[e]	10	*ZMIZ1*	.78 ± .05	.78 ± .06	.79 ± .08	.78 ± .06	0.0093	3.94E-05	0.029	49.5	0.14
cg19215199	10	*ZMIZ1*	.82 ± .04	.83 ± .04	.82 ± .06	.83 ± .04	0.0093	3.66E-08	0.002	94.3	<0.001
cg13527922	11	*F2*	.86 ± .02	.87 ± .02	.87 ± .02	.87 ± .02	0.0051	1.54E-08	0.001	81.9	0.004
cg24101979[e]	17	*NXN*	.81 ± .03	.77 ± .04	.80 ± .05	.78 ± .04	0.0072	8.95E-05	0.001	92.4	<0.001
cg26003785[f]	17	*NXN*	.94 ± .01	.96 ± .01	.97 ± .02	.96 ± .01	0.0038	9.53E-09	0.001	27.3	0.25

cg26283240[e]	17	*NXN*	.87 ± .03	.86 ± .03	.88 ± .04	.87 ± .03	0.0065	2.03E-05	0.024	85.6	<0.001
cg06004017[e]	22	*MN1*	.86 ± .02	.90 ± .02	.87 ± .03	.89 ± .02	0.0046	0.00019	0.048	74.6	0.02
cg20680669	22	*MN1*	.96 ± .02	.96 ± .02	.99 ± .01	.97 ± .02	-0.0049	2.09E-08	0.001	67.4	0.046

28-day Trailing average starts from the day of the visit. A complete list of all CpGs that meet genome-wide significance or FDR significance for 28-day $PM_{2.5}$ is provided in Supplemental Material, Excel File S1.

[a] CHR: chromosome

[b] Estimated difference in methylation for a 10 $\mu g/m^3$ increase in $PM_{2.5}$ adjusted for sex, age, income (education years for NAS, in which information on income was not available), smoking status, alcohol intake, BMI, temperature (moving average always matching with the PM exposure window), day of the week, season and the proportion of five estimated white blood cell types: Monocytes, B Cells, CD8 T Cells, CD4 T Cells, NK

[c] P-values, Bonferroni significance level at 7.5E-08

[d] FDR: False Discovery Rate with Benjamini-Hochberg method, significance level at 0.05

[e] Non-Bonferroni significant but FDR significant CpGs located in the a gene with a Bonferroni significant CpG

[f] Shown in Figure 3

[g] FDR significant and Bonferroni significant at 7-day $PM_{2.5}$

[h] NA: no annotated gene

FIGURES LEGENDS

Figure 1. Manhattan plots showing fixed-effect p-values from the meta-analysis of KORA F3, KORA F4 and NAS longitudinal cohort studies across the human genome after fully adjusted model. Each dot corresponds to a CpG methylation site. Panel A: 2-day $PM_{2.5}$ exposure; Panel B: 7-day $PM_{2.5}$ exposure; Panel C: 28-day $PM_{2.5}$ exposure ($\mu g/m^3$).

Figure 2. Forest plots (left side) and Regional plots regarding cg19963313 that achieved genome-wide significance level and cg02608596 that was close to genome-wide significance at 7-day average and showed homogeneity. Forest plots show KORA F3, KORA F4 and NAS longitudinal cohort estimates and pooled meta-analysis results. Regional plots show the p-values from Figure 1, Panel B of each annotated CpG sites (diamonds) in a 200k bp length genome segment around the top CpG. The color and the size of the diamonds represent the intensity of the correlation with the top CpG target (in the center). The blue broken line connects the average methylation value of adjacent CpG sites; the right axis displays the 0-1 methylation scale. Correlations and averages values are calculated as mean of the three studies. Green arrows represent gene extension.

Figure 3. Forest plots (left side) and Regional plots regarding the CpGs that achieved Bonferroni genome-wide significance level and homogeneity at 28-day exposure. Forest plots show KORA F3, KORA F4 and NAS longitudinal cohort estimates and pooled meta-analysis results. Regional plots show the p-values from Figure 1, Panel C of each annotated CpG sites (diamonds) in a 200k bp length genome segment around the top CpG. The color and the size of the diamonds represent the intensity of the correlation with the top CpG target (in the center). The blue broken line connects the average methylation value of adjacent CpG sites; the right axis displays the 0-1 methylation scale. Correlations and averages values are calculated as mean of the three studies. Green arrows represent gene extension. Orange outlined diamonds highlight FDR significant CpG sites.

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Figure 1.

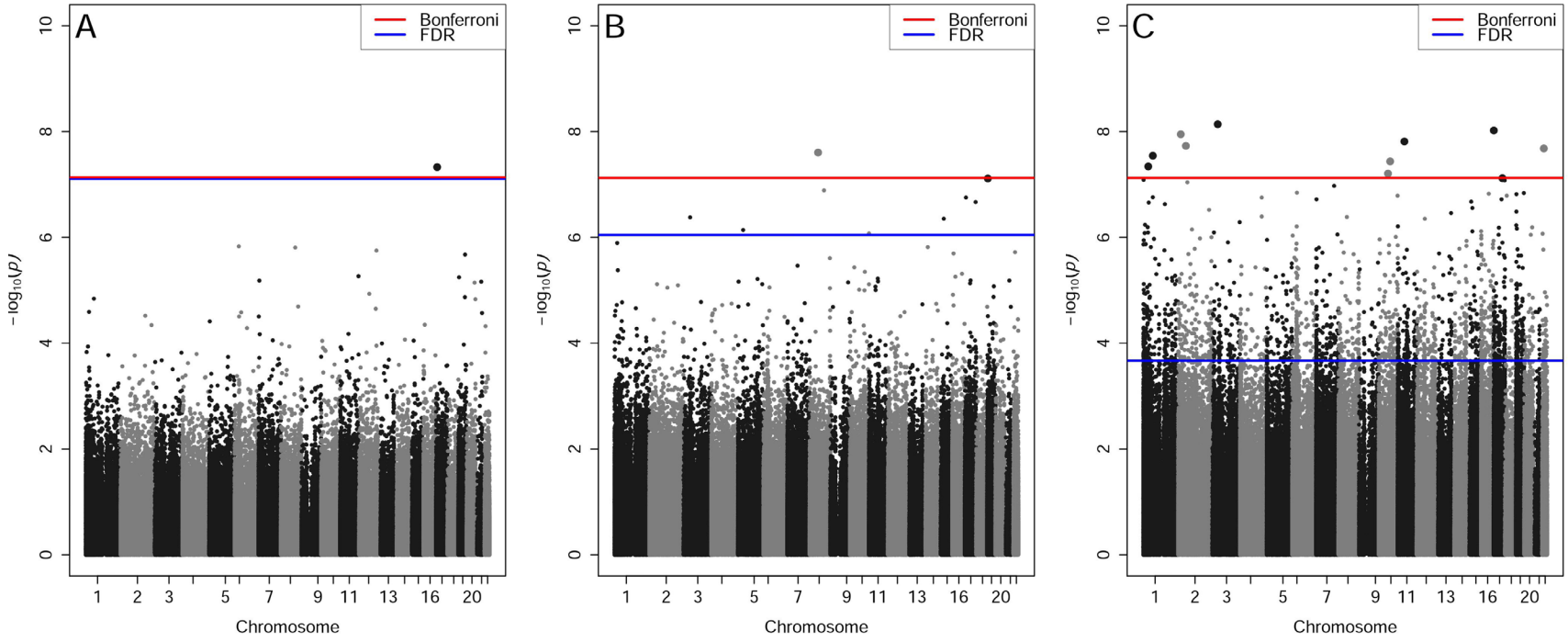

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Figure 2.

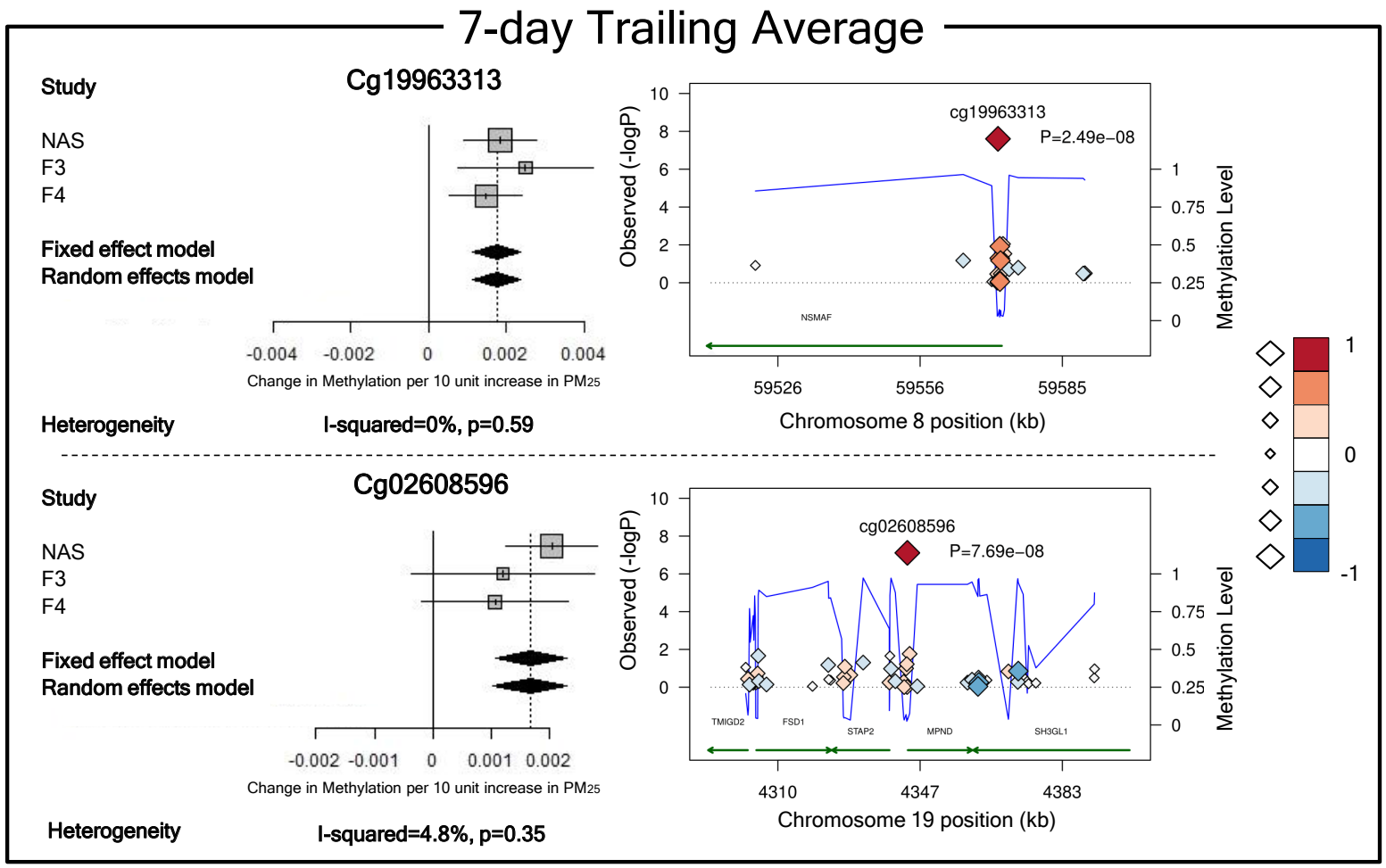

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Figure 3.